LABORATORY QUALITY-CONTROL STUDIES OF STERILITY IN BOLL WEEVILS PRODUCED FOR THE MISSISSIPPI PILOT ERADICATION EXPERIMENT

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LABORATORY QUALITY-CONTROL STUDIES OF STERILITY IN BOLL WEEVILS PRODUCED FOR THE MISSISSIPPI PILOT ERADICATION EXPERIMENT

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ABSTRACT

The Cotton Insects Physiology Laboratory, Baton Rouge, La., monitored the sterility of boll weevils (*Anthonomus grandis* Boheman) produced for mass release in a pilot eradication program in southern Mississippi. Weevils were reared and sterilized at the Robert T. Gast Laboratory, Mississippi State, Miss., during 1972 and 1973. Progressive increases in sterility coincided with improvements in the health and vigor of the weevils; an extension of the treatment period; the addition of a second chemosterilant, hempa, to the busulfan-treated diet; and refinements in the preparation and feeding of chemosterilant diets.

INTRODUCTION

In 1971 a pilot experiment was begun to attempt the eradication of the boll weevil (*Anthonomus grandis* Boheman) in southern Mississippi (*1*).[2] Several suppression techniques were employed, including the release of boll weevils sterilized with busulfan or busulfan plus hempa. These chemosterilant treatments had consistently induced a high level of permanent sterility in small-scale laboratory tests (*3, 5, 7*).

In this paper, we report on quality-control studies performed at Agricultural Research Service's (ARS) Cotton Insects Physiology Laboratory at Baton Rouge, La. Newly emerged weevils were fed chemosterilant-treated diet for 6 or 7 days at the Robert T. Gast Rearing Laboratory at Mississippi State, Miss. (*2*). Immediately upon termination of the treatment, samples of adults were shipped by air to Baton Rouge. We followed the scaling up of the treatment process, culminating with the pilot-plant production of about 2,700,000 sterile males during the summer of 1973.

During 1973 the ARS Boll Weevil Research Laboratory at Mississippi State conducted parallel assays of sterility in males produced at the Gast Rearing Laboratory. Their procedures differed somewhat from ours, and their study involved fewer insects. A summary of their data has been published elsewhere (*6*).

By "quality-control testing" we mean the frequent monitoring of sterility in samples of treated weevils under carefully standardized conditions. A high-quality weevil is one that is permanently sterilized and which is still capable of competing with wild untreated males. Overall competitiveness or effectiveness of sterile males in the field depends on a number of factors such as the ability to produce pheromone and motile sperm, the capacity to mate, the level of

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[2] Italic numbers in parentheses refer to items in "Literature Cited" preceding the appendix.

sterility, and mean longevity. We concerned ourselves only with the last two qualities, sterility and longevity; they are of prime importance and can be measured easily under routine laboratory conditions.

Unfortunately, at the present time there is no practical and reliable method for assessing the sterility of treated weevils prior to release. Therefore, we must follow the time-consuming practice of measuring sterility in samples of treated weevils, knowing that little information will be available until 2 to 3 weeks after the release date. However, if properly conducted, this type of laboratory work will provide information not obtainable by any other means. The information can be relied upon and corrective measures can be made with confidence prior to future releases.

In a large-scale release program certain errors can be detected by various monitoring procedures in time for corrective action to be taken. For example, if chemical analysis of chemosterilant-treated diet indicates improper mixing, the deficient batches may be discarded before use (James Frazier, unpublished data). Also, if microscopic examination of newly emerged weevils reveals high levels of microbial contamination, the decision may be made not to utilize them.

The quantitative measure of locomotor activity is another way to determine the general vigor and health of young adult weevils. Busulfan-induced sterility has been shown to be correlated with reduced activity, and locomotor tests can be utilized to predict, within limits, weevil sterility (*9*). Consistently high locomotor scores would indicate the need for increasing the sterilizing dosage. The greatest value of these early-warning tests lies in the fact that they alert us to possible deficiencies that require immediate attention; and, they indicate to us that we can anticipate substandard performance in the weevils being sterilized.

GENERAL PROCEDURES

The most meaningful laboratory assays of sterility are based on the examination of representative samples of weevils. The insects should be held for an extended period under conditions resembling those in the field. These general guidelines were followed in devising the standard procedures as described below:

1. We collected a representative sample of 50 to 300 weevils from several treatment cages.
2. No more than 25 to 50 weevils were placed in new 1-pint ice cream cartons.
3. The posttreatment temperature was maintained at a constant 30° C (85° F) with a 14:10 fluorescent light:dark schedule. Limited tests have shown that similar results are obtained regardless of whether weevils are held at a daily fluctuating temperature (21° to 32° C) or at a constant 30°.
4. Fresh squares were provided each day (8 squares/50 weevils) with adequate moisture. Artificial diet was substituted occasionally when squares were in short supply.
5. Dead insects were counted and removed daily.
6. Progeny development was used as a measure of sterility rather than egg hatch since dominant lethals may be expressed after hatch. Surface-sterilized eggs were implanted individually in a larval diet that permitted at least 50% adult emergence in the controls. A diet containing cottonseed meal or flour was found to be suitable (diet C in appendix).
7. To make certain that untreated females used for outcrosses were virgin, only those females taken directly out of pupation cells were used. All groups of untreated males and females were resexed before mating.

Assay of Male Sterility

We limited the mating frequency of males to no more than once per week. Treated males were placed with untreated virgin females at a sex ratio of 1:1 for a single 24-hour period within 7 days after termination of treatment. The sexes were separated, and 100 eggs from each group of females were implanted individually in larval diet. The females were saved for a second mating with surviving males on the 14th day, again at a sex ratio of 1:1. Sterility in males during the first week and at the end of 14 days was determined from the percentage of eggs that failed to develop into normal adults. Normal eggs were also implanted in larval diet as a check on the procedure. Sterility on the 21st day ("permanent sterility") was based on the percentage of survivors with atrophied testes as determined by microscopic examination. A male was not considered sterile unless all four lobes of the testes were

atrophied. Selected matings were also made on the 21st day to confirm the fact that weevils with atrophied testes were sterile.

Assay of Female Sterility

Our usual method for measuring female sterility was to allow treated females to mate with untreated males immediately after treatment. Eggs were counted for 6 or 7 days, and groups of up to 50 eggs were individually implanted daily in larval diet. Females were not considered to be effectively sterilized unless they became infecund within 7 days posttreatment. This test yielded a conservative measure of sterility since it indicated how many progeny might have been produced in the unlikely event that all treated females mated with fertile males at the time of release. Results were expressed as average progeny per treated female for the 6- or 7-day posttreatment period.

The 1972–73 pilot eradication experiment was to have involved the release of only sterile males. However, as one might expect, the manual separation of the sexes was not completely accurate. It is estimated that about 2% of the released weevils were females. In measuring the sterility of these females we decided not to remate them with untreated males. All had mated at some time during the course of the 6- or 7-day treatment. Accordingly, samples of females isolated at the end of the feeding period were held out of contact with males, and fecundity and egg viability were measured for periods up to 21 days.

RESULTS

Experimental data appear in chronological order, beginning with an evaluation of diet and sterile weevils produced in late 1972 and ending with the final large-scale treatments at the Gast Rearing Laboratory in August 1973. A description of methods and a discussion of results are included for each set of experiments.

Evaluation of Treated Weevils and Diet in 1972

Early rearing problems

Every effort was made to complete the Gast Rearing Laboratory and to install equipment well before the scheduled insect releases were to begin in early June of 1972. However, when the release program began, the facility had been largely untested, and the newly employed technicians were inexperienced. There was simply no time to make needed modifications and adjustments in equipment or to develop effective sanitation procedures. In spite of severe handicaps, the staff managed to produce about 160,000 sterile males per week during June and July of 1972.

Quality-control studies conducted at the Boll Weevil Research Laboratory at Mississippi State indicated that sterility in these early weevils was lower than desired and mortalities were often excessive. Beginning in late July a concerted effort was made by personnel from ARS, Mississippi State University, and the Animal and Plant Health Inspection Service to discover what corrective steps could be taken to improve the quality of the sterile weevils. At that time the general health and vigor of the weevils was impaired by microbial contamination of the gut. Apparently the eggs were not completely surface-sterilized with 0.8% formaldehyde, and the diet as well as the weevils became contaminated.[3]

Weevils in such a condition would be expected to consume less chemosterilant-treated diet and therefore would not receive a sterilizing dose. Also, the chemosterilant, busulfan, was not dispersed uniformly in the diet. Earlier laboratory studies had shown that a fine dispersal of busulfan was essential for the induction of high sterility.

Changes in the diet

In late July and early August of 1972 several changes were made in the composition and method of formulation of the busulfan-treated diet: Wesson oil (a mixture of cottonseed and soybean oils) was added to increase the level of essential fatty acids; potassium sorbate, methyl *p*-hydroxybenzoate, and erythromycin were added to retard the growth of micro-organisms, and corn sirup was used to suspend the ball-milled busulfan before it was added to the liquid diet. The corn sirup originally was to serve as a liquid carrier for busulfan that could be continuously metered into the diet as it came from

[3] P. Sikorowski, O. H. Lindig, J. M. Wyatt, and L. D. Coons. Boll weevil: Method for surface sterilization of eggs and the determination of bacterial contamination of adults. Manuscript in preparation.

the flash sterilizer. However, a metering device was never installed, and the busulfan was always added to weighed batches of diet. A ball-milled mixture of busulfan and powered cellulose was used to produce a fine free-flowing powder. The powdered busulfan was mixed with corn sirup and a small portion of liquid diet in a high-speed blender; this mixture was then stirred into the remainder of the liquid diet maintained at 55° C. This diet is referred to as formulation B in the appendix.

During the fall of 1972 we bioassayed over 30 samples of chemosterilant diet prepared at the Gast Laboratory. Nearly all of the batches of diet induced a high degree of sterility following a 6-day feeding period in small-scale tests at Baton Rouge.

Sterility in Late 1972 and Early 1973

Beginning in late October of 1972, we discontinued quality-control tests on diets and began determining sterility levels in weevils that had been reared and sterilized under large-scale conditions at the Gast Rearing Laboratory. Between October 20 and November 1, 22 groups of weevils were shipped to Baton Rouge. These weevils had been treated with 0.11% and 0.13% busulfan, with and without 0.4% hempa. All treatments produced 100% initial and permanent sterility in males, but unfortunately male mortalities were usually quite high, ranging between 80% and 100% by the third week after treatment.

Beginning on December 4, we evaluated additional groups of treated males. Weevils from these same groups were also sent to other laboratories for simultaneous physiological and biochemical assays. At Baton Rouge, we measured initial and 21-day sterility levels. For initial sterility, males were mated with virgin females, and 50 eggs collected during the first week posttreatment were implanted in larval diet. A group of 50 males was held for 3 weeks on fresh squares at 30° C, and the survivors were then dissected to determine permanent sterility (table 1). As in the earlier tests, most of the treatments completed in early December were too toxic. This was certainly true of the two series in which the treatments ended on December 4 and 5. However, in the series dated December 10, the lowest dosage resulted in a 21-day mortality of 70%, which is almost within the optimum range. In the treatment ending December 19, 0.08% busulfan produced nearly optimum results, and even some of the 0.06% busulfan treatments that followed gave satisfactory results. This decline in mortality that occurred on or after December 10 appears to be correlated with a change in the composition of the larval diet. We believe that some of the weevils used in the December 10 test were reared from the modified larval diet containing cottonseed meats (diet B in appendix); all weevils used in subsequent tests were reared from the modified diet. Weevils reared from the new diet produced more eggs and were more active than those reared from the old diet (D. Glover, unpublished data).

We still are not sure why the mortalities during November and early December were so high. We used 0.11% busulfan during the preceding month and obtained very good results. In October we decided to omit the soy protein (Promine D) from the chemosterilant diet. This change might have affected mortality and sterility. However, in an earlier cooperative test at Gulfport, Miss. (D. Glover and N. Earle, unpublished data 1972), we compared chemosterilant diets containing cottonseed meal and soy protein with diets containing cottonseed flour without soy protein; the results were essentially the same. The temperature during treatment was increased from about 76° to 80° F in September, well before we began to encounter higher mortalities in treated weevils, so this was not the deciding factor. Only two other changes in the procedures occurred during October—the chemosterilant diet pellets were shortened and the wax coating was more uniform.

Thus, all we can conclude is that unknown changes in early October resulted in higher sterility and mortality and that a change in the larval diet in December may have lowered mortality considerably.

Sterility of Weevils Produced for Experimental Release, Spring of 1973

In early 1973, seven groups of treated weevils were sterilized for release in small fields containing low populations of wild weevils. Each shipment consisted of males and females treated with busulfan alone as well as with a combination of busulfan plus hempa. The experiment

TABLE 1.—*Mortality and sterility in groups of 50 male weevils treated in late 1972 and early 1973*

Last day of treatment	Chemosterilant treatment (%)		Posttreatment condition on—			
			7th day		21st day	
	Busulfan	Hempa	Dead (%)	Sterile (%)	Dead (%)	Sterile (%)
			FED SQUARES			
1972						
Dec. 4	(1)	(1)	10	...	28	...
	0.08	0	40	100	76	100
	.1	0	48	94	94	100
	.11	0	82	100	100	100
	.1	.4	42	100	86	100
	(2)	(2)	14	...	18	...
Dec. 5	(1)	(1)	6	...	10	...
	.08	0	36	100	68	100
	.1	0	60	100	82	100
	.12	0	90	100	96	100
	.1	.4	62	100	96	100
	(2)	(2)	12	...	28	...
Dec. 10	(1)	(1)	6	...	22	...
	.08	0	18	100	70	93
	.1	0	20	100	66	100
	.12	0	46	100	88	100
	.1	.4	16	100	64	100
Dec. 19	.08	0	12	100	58	100
	.08	0	10	100	60	100
Dec. 21	.06	0	8	100	30	88
	.06	0	4	98	36	91
Dec. 26	.06	0	12	98	46	85
	.06	0	10	100	58	100
Dec. 28	.08	0	[3]14	[3]100	[3]40	[3]73
	.08	0	[3]4	[3]98	[3]24	[3]76
1973						
Jan. 7	.08	0	20	100	46	88
	.1	0	58	100	82	100
	.08	.4	20	100	44	100
	.1	.4	40	100	84	100
			FED ARTIFICIAL DIET			
Jan. 9[4]	[5]0.08	0	1	98	30	97
	.12	0	28	100	98	100
Jan. 15	(1)	(1)	4	...	24	...
	.08	0	10	100	54	95
	.1	0	4	100	72	100
	.12	0	18	70	90	100
	.1	.4	10	100	66	100
	(2)	(2)	4	...	32	...
Jan. 21[4]	.08	0	2	96	42	93
	.08	0	2	100	36	90
	.08	.4	2	100	46	100
	.10	0	4	100	54	100
	.10	0	6	100	48	100
	.10	.4	12	100	70	100
Jan. 23	.08	0	0	100	36	100
	.10	0	6	100	41	100
	.08	.4	8	100	32	100
	.10	.4	0	100	32	100

See footnotes at end of table.

TABLE 1.—*Mortality and sterility in groups of 50 male weevils treated in late 1972 and early 1973*—Continued

Last day of treatment	Chemosterilant treatment (%)		Posttreatment condition on—			
			7th day		21st day	
	Busulfan	Hempa	Dead (%)	Sterile (%)	Dead (%)	Sterile (%)
	FED ARTIFICIAL DIET—Continued					
Jan. 25	.08	0	8	100	58	95
	.08	0	4	100	40	100
	.10	0	10	100	60	100
	.10	0	6	100	64	100
	.08	.4	0	100	26	100
	.10	.4	4	100	70	93
Jan. 26	.1	.4	0	100	76	100
	.1	.4	0	100	40	100
	.1	.4	0	100	48	100
	.1	.4	0	100	40	100
Jan. 29	.06	0	4	100	22	97
	.08	0	4	100	34	97
	.10	0	1	100	48	100
Jan. 30[4]	.08	0	2	100	62	90
	.10	0	4	100	70	100
	[6].10	0	2	98	46	100
Feb. 1	[6].10	0	2	100	46	100
Feb. 5	.06	0	0	...	56	86
	.08	0	4	100	50	100
Feb. 6	.08	0	18	100	32	100
	FED SQUARES					
Feb. 7[4]	([1])	([1])	1	...	3	...
	0.10	0	2	100	66	100
	.06	.4	0	100	34	100
	.08	.4	6	100	22	100
	([1])	([1])	0	...	18	...
	.08	0	8	100	16	71
	.10	0	10	100	34	...
Feb. 12	.08	0	...	...	49	72
	.08	0	...	...	42	79
Feb. 13	([1])	([1])	...	...	41	...
	.08	0	...	...	43	82
	.08	0	...	...	65	77
Feb. 14	([1])	([1])	...	...	30	...
	.08	0	...	...	38	83
	.08	0	...	...	41	78
Feb. 15	.08	.4	...	...	38	100
	.1	.4	...	...	61	89
Feb. 26	([1])	([1])	12	...	18	...
	.06	0	8	98	30	61
	.08	0	12	100	38	77
	.10	0	50	100	78	100
	.08	.3	18	100	46	100
	.08	.4	34	100	70	100
	([2])	([2])	4	...	28	...

[1] Control (no chemosterilant treatment). The average adult emergence from eggs obtained from 11 groups of untreated weevils between December 4 and December 26 was 57%. [2] Diseased. These groups consisted of adults reared from larval diet that had been deliberately contaminated with a culture of bacteria isolated from the rearing laboratory. [3] Treatment period was 5 days instead of 6 days. [4] Approximated date. [5] Plus meat. [6] Plus promine.

was designed to determine whether it would be safe to release females as well as males treated with either busulfan or busulfan plus hempa.

The busulfan-hempa combination treatment was more effective against males than the busulfan treatment. A mixture of 0.09% busulfan plus 0.4% hempa appeared to be optimum for consistently high sterility. A dosage of 0.08% busulfan alone appeared to be too low. The data are summarized in table 2.

The results for females (not given in table 2) showed that hempa reduced progeny development, but not as much as was desired. Females treated with the combination of busulfan plus hempa produced an average of 0.26 progeny per female when outcrossed with normal males, as compared with 1.4 progeny for the females treated with busulfan only. In earlier small-scale laboratory tests, females treated with busulfan plus hempa produced only 0.02 progeny per female (*3*).

Sterility in Weevils Treated for Release in the Pilot Eradication Experiment, Summer of 1973

Male sterility

Chemosterilant treatments were sharply curtailed during March, April, and May of 1973 because of a shortage of funds. This was unfortunate because the facility had not been adequately tested under anticipated production conditions and some of the new personnel were still relatively inexperienced. Also, we had planned on making final adjustments in the methods for chemosterilant-diet formulation and feeding.

Sterility was determined earlier in samples of every group of males treated for release beginning in late May and ending in early August of 1973 (table 3). Samples of males were also assayed for sterility twice weekly at the Boll Weevil Research Laboratory from May through the end of July (*6*). Consistently higher sterility levels were obtained after June 14 as a result of increasing the treatment period from 6 days to 7 days for most of the groups. The decision to extend the treatment period by 1 day was based on standardized locomotor tests run on each group of treated weevils at the Boll Weevil Research Laboratory. In retrospect, we probably could have obtained the same effect by increasing the concentration of busulfan to 0.1% and by limiting the treatment period for all weevils to only 6 days.

In June, concern was expressed over the possibility that the few treated females released

TABLE 2.—*Initial and 21-day mortalities and sterility levels in groups of 50 males treated for 6 days for experimental release, spring of 1973*

[All groups fed squares]

Last day of treatment	Chemosterilant treatment (%)		Posttreatment condition on —			
			7th day		21st day	
	Busulfan	Hempa	Dead (%)	Sterile (%)	Dead (%)	Sterile (%)
Feb. 26	0.08	0	8	[1]94	38	68
	.08	.3	10	[1]100	20	94
Mar. 6	.08	0	8	[1]94	23	83
	.08	.3	6	[1]98	30	87
Mar. 12	.08	0	[2]32	...	78	100
	.08	.3	[2]58	...	82	100
Mar. 19	.08	0	6	100	21	76
	.08	.4	10	100	39	89
Mar. 26	.08	0	24	100	59	84
	.08	.4	24	94	43	96
Apr. 2	.09	0	36	100	65	100
	.09	.4	36	100	83	88
Apr. 9	.09	0	32	100	66	100
	.09	.4	24	100	62	100

[1] Sterility based on egg hatch; actual sterility should be greater since some larvae fail to develop.

[2] Weevils were in poor condition because of high temperature during treatment and delays during shipment to Baton Rouge.

TABLE 3.—*Mortality and sterility levels in samples of males treated for release in Mississippi during the summer of 1973*

Last day of treatment	Length of treatments (days)	Weevils in sample	Posttreatment mortality (%) on—			Posttreatment sterility (%) on[1]—		
			7th day	14th day	21st day	7th day	14th day	21st day
			TREATED WITH 0.09% BUSULFAN					
May 31	6	100	38.0	53.0	80.2	100	97.0	70.6
June 3[2]	6	100	41.0	73.0	84.8	96.9	100	80.0
June 5	6	100	...	82.0	86.0	100	100	85.7
June 6	6	100	...	72.0	88.8	100	100	100
June 7	6	100	44.0	75.0	82.8	100	100	82.4
June 8	6	100	47.0	71.0	76.2	94.0	86.0	96.0
June 8	6	100	11.0	56.0	66.0	99.0	100	97.1
June 9	6	100	33.0	61.0	84.5	100	98.0	100
June 10	6	100	19.0	50.0	73.0	99.0	72.0	83.3
June 11	6	100	33.0	55.0	71.9	99.5	100	92.0
June 12	6	100	45.0	55.0	75.9	100	78.0	85.0
June 14	7	100	46.0	62.0	74.2	100	100	95.8
June 15	7	100	27.0	65.0	73.7	100	100	96.0
June 16	7	100	37.0	62.0	76.3	100	100	82.6
June 18	8	100	63.0	78.0	93.4	100	100	100
June 18	7	100	63.0	74.0	89.5	100	100	100
June 19	7	100	55.0	73.0	88.3	99.0	98.0	100
June 19	6	100	39.0	75.0	88.4	100	100	83.3
June 21	7	100	59.0	73.0	85.6	100	100	92.9
June 22	7	100	67.0	84.0	93.0	100	100	85.7
June 23	7	100	52.0	75.0	87.0	100	100	100
June 24	7	100	38.0	65.0	79.8	100	100	100
June 24	6	100	69.0	81.0	82.1	100	100	100
June 25	6	100	42.0	57.0	74.0	97.0	92.0	88.0
June 27	7	100	56.0	67.0	75.5	100	100	96.0
June 27	6	100	54.0	72.0	79.8	100	100	89.5
June 29	7	100	74.0	82.0	87.9	100	100	100
June 30	7	100	29.0	77.0	82.7	100	100	100
July 2	7	100	67.0	80.0	81.7	100	100	100
July 3	7	100	75.0	86.0	92.9	100	100	100
July 4	7	100	46.0	64.0	75.5	100	100	100
July 5	7	100	45.0	66.0	78.2	100	100	100
July 5	6	100	54.0	77.0	86.6	100	100	100
July 7	7	100	41.0	79.0	85.9	100	100	100
July 9	7	100	65.0	77.0	80.0	100	99.0	95.0
July 10	7	100	55.0	70.0	79.6	100	99.0	95.0
July 11	7	100	71.0	78.0	86.0	100	100	85.7
July 12	7	150	63.3	72.7	82.9	100	100	96
July 13	7	100	57.0	72.0	78.8	100	100	95.2
July 14	7	100	74.0	86.0	90.0	100	100	100
July 14	6	100	55.0	68.0	88.3	100	100	100
July 14	7	300	50.6	71.3	84.4	100	100	97.9
July 17	7	300	47.0	70.0	86.2	100	100	100
July 18	7	300	24.7	81.7	94.7	100	100	100
July 19	7	300	44.7	74.3	87.7	100	100	97.2
			TREATED WITH 0.09% BUSULFAN AND 0.4% HEMPA					
July 20	7	100	49.0	25.7	84.5	100	100	100
July 21	7	300	51.0	72.0	87.5	100	100	97.1
July 22	7	100	21.0	21.3	75.0	100	100	100
July 23	7	250	54.4	71.6	87.3	100	100	100
July 24	7	300	52.0	72.3	86.3	100	94.0	100

See footnotes at end of table.

TABLE 3.—*Mortality and sterility levels in samples of males treated for release in Mississsippi during the summer of 1973*—Continued

Last day of treatment	Length of treatments (days)	Weevils in sample	Posttreatment mortality (%) on—			Posttreatment sterility (%) on[1]—		
			7th day	14th day	21st day	7th day	14th day	21st day
			TREATED WITH 0.09% BUSULFAN AND 0.4% HEMPA—Continued					
July 25	7	300	64.0	83.0	91.1	100	100	96.3
July 26	7	300	67.7	80.3	88.8	100	100	100
July 27	7	300	53.0	71.7	84.3	100	100	97.8
July 28	7	300	39.3	68.0	84.6	100	100	100
July 29	7	300	63.3	92.7	93.4	100	100	100
July 30	7	300	68.7	78.3	88.5	100	100	100
July 31	7	300	63.0	84.0	91.6	100	100	100
Aug. 2	7	300	29.0	73.7	81.8	...	100	100
Aug. 3	7	300	47.0	73.0	82.4	100	100	100
Aug. 4	7	300	46.7	77.7	84.6	100	100	100
Aug. 5	7	300	46.0	74.7	83.7	100	100	100
Aug. 6	7	300	63.3	75.7	88.9	100	100	93.5
Aug. 7	7	300	56.3	73.3	82.7	100	100	96.2
Aug. 8	7	300	63.7	80.3	86.6	...	100	100
Aug. 9	7	300	60.0	71.6	82.7	100	100	98.1
Average	...	...	51.3	73.9	85.0	99.7	98.7	95.7

[1] The average development for 12 groups of eggs obtained from untreated weevils was 52%.
[2] Approximate date.

along with the males were not completely sterile and would contribute to the field population. The 0.4% hempa that was subsequently added to the treated diet to increase female sterility probably also increased male sterility. All weevils released after about July 19 were treated with a mixture of busulfan and hempa.

It is our conclusion that satisfactory levels of sterility were maintained in male weevils for at least 3 weeks after the termination of the 6- or 7-day treatment period. Sterility levels in a few of the groups were low during the first 2 weeks of operations, but improved as treatment methods became more standardized and more effective throughout the summer. For example, the average 1- and 3-week sterility values increased progressively as shown in table 4. Thus, we found no evidence for any decline in the quality of sterile insects being produced toward the end of the summer of 1973.

TABLE 4.—*Sterility of males treated during the summer of 1973*

Release dates	Sterility (%) on—	
	7th day posttreatment	21st day posttreatment
May 31–June 11	98.8	88.7
June 14–July 5	99.8	95.2
July 9–July 27	100	98.0
Aug. 2–Aug. 6	100	98.8

The average sterility of males at 3 weeks (95.7%) is probably a conservative estimate; in about 2% of the survivors, all four lobes of the testes appeared normal, and single pair matings confirmed the fact that these males were fertile. However, the remaining 2% classified as fertile had only one or two functional lobes, and it was determined from limited single pair matings that these males were still transferring sterile sperm; subsequent matings would eventually result in fertile offspring as new fertile sperm gradually replaced the sterile sperm.

If it is assumed that laboratory mortality rates are reasonable approximations of those in the field, we may calculate a rough estimate of the overall sterility level in the field: 49% of the males survived for 1 week and were 99.7% sterile; 26% survived for 2 weeks and were 98.7% sterile; and 15% survived for 3 weeks and were 95.7% sterile. These data can be applied to a hypothetical field situation to estimate sterility in the field at any given time. For example, let us apply these data to a 10-acre field in which sterile males are being re-

leased at the rate of 100 males per acre per week. For every 1,000 males released, there would be 150 survivors 3 weeks after release, 260 survivors 2 weeks after release, and 490 survivors 1 weeks after release. Of the 150 males which survived 3 weeks, 6.45 would be expected to be fertile [150× (100—95.7% sterility)]; of the 260 males surviving 2 weeks, 3.38 would be expected to be fertile; and of the 490 males surviving 1 week, 1.47 would be expected to be fertile. So, of the total of 3,000 males released, 900 survivors would be present in the field immediately prior to the next 1,000 males released (assuming no males survived past 3 weeks). Of these 900 males 11.3 would be expected to be fertile, so we could estimate a 98.74% level of sterility immediately prior to the fourth release of 100 males. This would be the lowest level of sterility expected to occur in the field at any given time. Immediately after the new release of 1,000 males (assume 100% initial sterility), there would be 1,900 males in the field; 11.3 of these would be expected to be fertile. This amounts to a 99.39% level of sterility, the highest that could be expected to occur in the field. So at any given time overall sterility in the field-released weevils could be estimated to be between 98.4% and 99.39%. If releases were made more frequently than once a week, overall sterility would be slightly higher; if they were made less than once a week overall sterility would be slightly lower.

Although the condition of the weevils produced in 1973 was vastly superior to that of the weevils reared in 1972, mortalities in control and treated weevils were still rather high in 1973, and we have no adequate explanation for this. For example, average mortalities for eight samples of untreated weevils after 1, 2, and 3 weeks were 22%, 35%, and 40%, respectively. The mortality data do not include losses that occurred during the 6- or 7-day feeding period. We estimate that 15%–20% of the controls and about 30% of the treated weevils died or were lost during this period. Extra handling during the separation of sexes was unavoidable and probably contributed to the mortality. Fortunately, those weevils that did survive appeared healthy and fed well on squares—an indication that they were producing enough pheromone to compete with wild males in the field.

Female sterility

Manual separation of male and female boll weevils proved to be quite accurate; it is estimated that sexed weevils returned to the rearing facility for treatment included an average of only 2% females. Nonetheless, it was feared that the release of these small numbers of females in the field might contribute to an increase in field populations if they were not effectively sterilized by the busulfan treatment. Accordingly, samples of females were isolated following treatment and were held out of contact with treated or untreated males. Eggs were collected and implanted in larval diet to determine whether the individual groups contained fertile individuals.

Fifteen groups of females, consisting of a total of 237 weevils, were isolated and shipped to Baton Rouge between June 28 and August 2. Out of this total, only four females (1.7%) were capable of producing any progeny without mating with a fertile male. Three out of these four weevils died by the third week. Other females continued to lay eggs after the third week but were sterile as a result of having mated with sterile males during the 6- or 7-day treatment period. It was concluded that an average of only about 2% of the groups of 50 weevils released per acre would contain a partially fertile female. It is very likely that these few females would mate with sterile males soon after release in the field and would then produce only sterile eggs. Detailed data are included in table 5.

We can think of at least one possible explanation for the relatively high fecundity of these treated females. Since the females were greatly outnumbered by the males, it is very likely that they mated during the first 2 or 3 days of treatment before the males were completely sterilized. During the next 3 or 4 days the females may have been continually harassed by males. Under such conditions the females would not consume very much chemosterilant-treated diet, and many would be expected to refuse to mate again with sterile males before release. Paradoxically, one would expect higher female sterility with more females present, and this is what we found among the groups consisting of equal numbers of males and females treated for experimental release in northern Mississippi (see next section).

TABLE 5.—*Sterility of females isolated from treatment cages containing a preponderance of males, summer of 1973*

Last day of treatment	Number of females per sample	Total eggs	Duration of test (days)	Total progeny	Number of fertile females	Days for egg count to reach 0
June 29	3	13	10	0	0	6
June 30	22	28	10	0	0	7
July 2	25	25	8	0	0	6
July 3	22	26	8	0	0	8
July 4	7	138	15	48	1	>15
July 5	4	11	7	0	0	6
July 9	25	382	21	1	0	>21
July 13	10	29	8	0	0	6
July 25	17	1	5	0	0	1
July 26	18	92	21	9	2	>21
July 27	18	88	17	10	1	15
July 28	16	44	7	0	0	6
July 29	16	3	6	0	0	4
July 30	14	4	4	0	0	3
July 30	20	6	5	0	0	5
Total	237	890	...	68	4	...

TABLE 6.—*Sterility of males and females treated with 0.09% busulfan + 0.4% hempa in July and August of 1973 for small-scale releases at Stoneville, Miss.*

Last day of treatment	Average progeny per treated female	Males (%) dead after—			Male sterility at 3 weeks post-treatment (%)
		1 week	2 weeks	3 weeks	
July 10	[1]1.4	57	75	81	100
July 22	0	40	52	76	100
July 24	.02	27	62	77	96
July 29	.02	...	...	...	...
July 31	.08	45	63	71	100
Aug. 6	0	48	61	76	96
Aug. 13	0	45	68	85	93
Aug. 15	0	48	68	82	100
Average	.19	44	64	78	97.9

[1] This was the only group in which egg production did not reach zero during the observation period; based on egg counts, we estimate that only 1 out of the original group of 50 females in this group was still fecund after 7 days.

Sterility of Weevils Treated As Mixed Sexes for Release in Northern Mississippi, July and August 1973

Towards the end of the 1973 season, several groups of weevils were treated as mixed sexes for experimental release outside the eradication zone. The purpose of the experiment was to determine what impact the release of treated females and males would have on the natural population. Releases were made in adjacent plots of cotton with low natural populations of weevils.

The treated diet was the same as that used during the latter part of the pilot program (0.09% busulfan + 0.4% hempa), but the feeding period was kept constant at 6 days. We limited quality-control tests to weekly mortalities of groups of 100 treated males, and a single sterility determination of males by microscopic examination of the testes at 3 weeks posttreatment. Groups of 50 females were outcrossed to

normal males, and egg production and progeny development were recorded for 1 week. Results for both sexes are given in table 6.

Interestingly, the mean 3-week male sterility (97.9%) for these groups was almost identical with the comparable mean value for the pilot-experiment groups treated for 7 days. Except for the first group, fertility in the treated females was reduced to an acceptable level.

These weevils were the last groups sterilized at the Gast Rearing Laboratory in 1973. It should be pointed out that our data do not give any indication of a decline in the quality of the sterile insects being produced at this time.

INTERPRETATION OF QUALITY-CONTROL DATA

It is obvious that sterility is directly related to the intensity and duration of treatment with sterilizing agents. However, there are a number of other important variables that must not be overlooked. Sterility may decline over a period of 1 to 3 weeks as unaffected germ cells give rise to fertile sperm. The rate of appearance of these new sperm in the ejaculate will depend on the number of surviving germ cells, frequency of mating, posttreatment temperature, and diet. Also, data on sterility do not mean much in the absence of data on longevity, and it is important to remember that temperature, crowding, nutrition, and sanitation will affect survival. All of these factors must be considered in devising quality-control procedures that will provide meaningful estimates of sterility and longevity in released weevils.

A temperature of 30° C (85° F) is probably representative of the mean summer temperature in southern Mississippi, and we have used it as the standard posttreatment temperature. If it is a little high, it simply means that our survival data are on the conservative side.

There is evidence that feeding on squares as opposed to artificial diet will allow a greater recovery of fertility (*4*). Whenever possible we have used squares for posttreatment feeding. In feeding artificial diet after treatment we have found that mortality as well as the sterility is often increased. When changing to square feeding, one must increase the chemosterilant dose by about 10% in order to produce the equivalent sterility and mortality. Spermatogenesis is a continuous process in adult males, requiring 11 days for the formation of mature sperm in normal males. We held the treated males for 21 days before examination of the testes in order to allow for any delay in the initiation or slowing down of the process of spermatogenesis attributable to chemosterilant action. If males contained spermatocytes or sperm bundles after 21 days, they were considered to be fertile.

Chemosterilant treatment of a given male does not always produce an all-or-nothing effect. It is not uncommon to find males with one or more of the four lobes of the testes atrophied along with functional lobes containing cells at all stages of development. We have classified any male with one or more functional lobes as "fertile," although under certain conditions such a male would still be capable of inseminating at least one female with sterile sperm. The time of appearance of the newly formed fertile sperm in the ejaculate would depend on how long ago spermatogenesis began, the rate of formation of sperm, and the frequency of mating (rate of sperm utilization). For example, a treated male dissected only 2 weeks after treatment and found to contain sperm bundles should probably still be considered sterile if it were to mate only once. We have found that 3 weeks after treatment, males with only one or two functional lobes are largely sterile. For example, we allowed sixty 3-week-old treated males to mate individually with virgin females and then immediately dissected them. The majority had completely atrophied testes and produced no progeny. One male had one recovered lobe, and a second male had two recovered lobes; less than 10% of the eggs from the two females mated to these males developed into adults. The two males were potentially fertile, and had they mated again after a suitable interval, they would have produced more fertile offspring.

It is important that mating frequency be controlled in laboratory quality-control studies in order to obtain valid data. If both sexes are to be released, we can only guess as to how often the males will be called upon to mate. However, if males only are released at a high ratio of released : wild weevils as during the 1972–73 pilot experiment, one would expect a very low mating frequency. In fact, at a ratio of 50 released males to 1 or 2 wild females, it seems unlikely that few, if any, of the released males would have the opportunity to mate more than

once or twice in their lifetime. We assume that there are no exceptionally attractive males in the released groups that would be approached repeatedly by females. In support of this assumption are olfactometer studies that have shown that individual untreated laboratory-reared males produce nearly uniform amounts of pheromone (E. Villavaso, unpublished data).

In order to simulate field conditions, laboratory tests should utilize infrequent matings, such as one 24-hour pairing per week. Continuous matings between sterile males and normal females are to be avoided since they tend to yield erroneous data biased in the direction of low sterility. For example, it is known that males and females held in close confinement will mate at least once per day. This being the case, permanently sterilized males would eventually become aspermic, possibly within 7 to 10 days, while a few incompletely sterilized males might begin to produce fertile sperm. The aspermic sterile males would be no match for the few fertile males. The fertile males could inseminate many females, the aspermic males, none. As a result, the apparent sterility as determined by egg hatch or progeny development would decline the longer the holding period.

Granted that sterile males should not be left continuously with females, there is still a question as to how long males and females should be left together for the controlled weekly matings. We selected 24 hours since Mitchell and Cross (*8*) found that some pairs remained together on the same cotton plant overnight where they had the opportunity to mate more than once. It is unlikely that males would actually inseminate females more than once or twice during this period.

Although we did not measure sterility in individual released males recovered from the field, a recommended procedure would be to place them with virgin females for a single period not to exceed 24 hours before being dissected.

There are additional precautions that must be observed in order to obtain valid information regarding sterility. One of the most serious sources of error is an undetected mistake in distinguishing the sexes during the isolation of virgin females for outcrossing with sterile males. The accidental introduction of a single fertile male when crossing sterile males with untreated virgin females would result in a significant apparent reduction in sterility. This would be especially true if the sterile males had been allowed to mate frequently and were becoming aspermic. Care must also be taken when examining the testes of 3-week-old treated males. Sterile males that have mated only once, or not at all, sometimes have nonfunctional testes that are enlarged because of densely packed sterile sperm. All questionable testes should be examined under high magnification to determine whether or not there are presperm stages present. Another possible source of error is introducing, as virgins, females which had mated prior to isolation. All virgin females should be isolated from pupal cells.

As stated earlier, the development of normal adults is a more accurate measure of egg fertility than hatch data. Data on the development of eggs from normal females should always accompany the data for eggs from sterile matings. Sometimes the average development in the controls is on the order of only 25% or 30%. And, it may be asked whether or not the percentage development for the treated groups should be multiplied by an appropriate factor, such as 2 or 3, in order to obtain a more meaningful measure of fertility. This should probably be considered, especially if significant numbers of eggs from test matings of sterile males show development. Adult emergence in our controls was consistently greater than 50%, which is probably within the normal range of development in the field (see footnotes to tables 1 and 3). Even if we were to increase all emergence values by a factor of 1.5, bringing the emergence of the control insects up to 75%, it would have little effect on the quality-control data for the 1973 pilot experiment. The mean sterility during the first week posttreatment would be 99.5% instead of 99.7%, and sterility after 2 weeks would be 98.0% instead of 98.7%. The third-week values would be unaffected since they were based on microscopic examination of the testes.

CONCLUSIONS

Progress in scaling up the chemosterilant treatment procedure was followed by quality-control studies of sterility induced by feeding busulfan- and hempa-treated adult diet. The quality of male weevils reared and treated at the Robert T. Gast Rearing Laboratory at Mississippi State improved greatly between June

of 1972 and August of 1973. Progressive increases in sterility coincided with improvements in the health and vigor of the weevils, an extension of the treatment period, the addition of a second chemosterilant, hempa, to the busulfan-treated diet, and with refinements in the preparation and feeding of chemosterilant diets. The overall mean sterility for the 1973 season was estimated to be between 98.74% and 99.39%. These figures were based on calculations of weighted averages of laboratory sterility values for males that survived 1, 2, and 3 weeks posttreatment. They take into account the fact that percentage sterility is always highest soon after treatment and that a recovery of fertility occurs only after many of the males have died. We considered 98% to be an acceptable level of sterility in view of the state of the art at the time. However, mortalities were excessive, reaching 50% by the end of the first week posttreatment. We believe that much of the mortality was caused by the frequent but necessary handling of the weevils as they were sexed and fed.

A small percentage of the few females that were treated and released along with the males showed some residual fertility. We have suggested that the very high male:female ratios during treatment inhibited normal feeding by the females. The females may have mated with males before the latter had become sterile; these females may have then been prevented from consuming adequate amounts of chemosterilant-treated diet because of frequent harassment on the part of the many males present. The interpretation and significance of quality-control data were discussed. The selection of the proper mating frequency for treated males was emphasized.

LITERATURE CITED

(1) Boyd, F. J. 1976. Operational plan and execution of the Pilot Boll Weevil Eradication Experiment. *In* Boll Weevil Suppression, Management, and Elimination Technology. Proceedings of a Conference, Feb. 13–15, 1974, Memphis, Tenn. U.S. Dep. Agric., Agric. Res. Serv. [Rep.] ARS–S–71.

(2) Davich, T. B. 1976. Boll weevil sterility. *In* Boll Weevil Suppression, Management, and Elimination Technology. Proceedings of a Conference, Feb. 13–15, 1974, Memphis, Tenn. U.S. Dep. Agric., Agric. Res. Serv. [Rep.] ARS–S–71.

(3) Flint, H. M., Earle, Norman, Eaton, Judy, and Klassen, Waldemar. 1973 Chemosterilization of the female boll weevil. J. Econ. Entomol. 66: 47–53.

(4) Hedin, P. A., Wiygul, G., Vickers, D. A., Bartlett, A. C., and Mitlin, N. 1967. Sterility induced by tepa in the boll weevil: Effective dose and permanency, gonadal changes, and biological turnover of labeled compound. J. Econ. Entomol. 60: 209–214.

(5) Klassen, W., and Earle, N. W. 1970. Permanent sterility induced in boll weevils with busulfan without reducing production of pheromone. J. Econ. Entomol. 63: 1195–1198.

(6) Lloyd, E. P., McCoy, J. R., and Haynes, J. W. 1976. Release of sterile male boll weevils in the Pilot Boll Weevil Eradication Experiment in 1972–73. *In* Boll Weevil Suppression, Management, and Elimination Technology. Proceedings of a Conference, Feb. 13–15, 1974, Memphis, Tenn. U.S. Dep. Agric., Agric. Res. Serv. [Rep.] ARS–S–71.

(7) McHaffey, D. G., Flint, H. M., Haynes, J. W., Klassen, W., Mitlin, N., and Davich, T. B. 1972. Sterility induced in boll weevils by alkylating agents administered in an adult diet. J. Econ. Entomol. 65: 13–19.

(8) Mitchell, H. C., and Cross, W. H. 1971. Mating of boll weevils in the field. J. Econ. Entomol. 64: 773–774.

(9) Wiygul, G., and Haynes, J. W. 1974. A locomotor test for the prediction of sterility in boll weevils. Entomol. Exp. Appl. 17: 452–453.

APPENDIX.—COMPOSITION OF LARVAL AND ADULT DIETS IN USE FROM AUGUST 1972 THROUGH AUGUST 1973

Component	A Larval diet, Gast lab (%)	B Chemosterilant diet, Gast lab (%)	C Larval diet, Baton Rouge lab[1] (%)
Water	79.10	82.00	85.00
Agar	1.55	1.45	1.62
Sucrose	1.74	3.10	3.24
Cottonseed meal or flour	6.60	5.30	4.86
Cottonseed meats (wet weight)	[2]6.97	0	0
Soy protein	2.54	[3]2.80	1.94
Cellulose powder	0	0	0
Salt mixture W	.26	.53	.24
Wesson oil	0	.28	0
Wheat germ oil	0	0	.32
Cholesterol	.075	.022	.065
Brewer's yeast or yeast extract	.87	0	.65
Ascorbic acid	.062	.36	.16
Vitamin mixture[4]	.009	.017	.023
Choline chloride	0	.043	.041
Sodium	0	.036	0
Potassium sorbate	.134	.092	0
Sorbic acid	0	0	.081
Methyl *p*-hydroxybenzoate	.087	.092	.081
Erythromycin	0	.0015	0
HCl	.062	0	0
Corn sirup	0	3.80	0
Busulfan[5]	0	0.06–0.13	0

[1] The larval and adult diets at Baton Rouge were identical except for reduced amounts of sorbic acid in the adult diet.

[2] Processed cottonseed meats contained about 50% moisture. This preparation was normally used only in the adult oviposition diet. However, all weevils emerging after Dec. 10 to 12, 1972, were reared from diet A containing cottonseed meats.

[3] The use of soy protein (Promine D) in the chemosterilant diet was discontinued in October 1972.

[4] The vitamin mixture consisted primarily of inositol with smaller amounts of niacinamide, calcium pantothenate, riboflavin, thiamine hydrochloride, pyridoxin hydrochloride, folic acid, biotin, and vitamin B_{12}.

[5] Weevils emerging after about July 13, 1973, were given 0.4% hempa in addition to 0.09% busulfan.

* 1976-GPO/1750-S/671=583/73

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